Antitumor properties of diastereomeric and geometric analogs of vitamin D_3

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Analogs of 1,25-dihydroxyvitamin D_3 with a reversed configuration at C-1 or C-24 and *E* or *Z* geometry of the double bond at C-22 in the side chain or at C-5 in the triene system were examined for their antiproliferative activity *in vitro* against a spectrum of various human cancer cell lines. The analogs coded PRI-2201 (calcipotriol), PRI-2202 and PRI-2205, such as calcitriol and tacalcitol (used as a referential agents), revealed antiproliferative activity against human HL-60, HL-60/MX2, MCF-7, T47D, SCC-25 and mouse WEHI-3 cancer cell lines. The toxicity studies *in vivo* showed that PRI-2202 and PRI-2205 are less toxic than referential agents. Even at total doses of 2.5–5.0 mg/kg distributed during 5 successive days, no changes in body weight were observed. Calcitriol and tacalcitol showed toxicity in the same protocol at 100 times lower doses. Calcipotriol was lethal to all mice after administration of a total dose of 5.0 mg/kg. The analog PRI-2205 appeared to be more active in mouse Levis lung cancer tumor growth inhibition than calcitriol, calcipotriol or PRI-2202. This analog did not reveal calcemic activity at doses which inhibit tumor growth *in vivo* nor at higher doses. *Anti-Cancer Drugs* 18:447–457 © 2007 Lippincott Williams & Wilkins.

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Introduction

The physiological level of the steroid hormone 1,25-$(OH)_2D_3$ [1,25-dihydroxyvitamin D_3 (calcitriol) – the most potent metabolite of vitamin D_3] is important mainly in the regulation of calcium homeostasis and bone metabolism [1], and also in prevention of cancer. Epidemiological data suggest that a higher incidence of some cancers, such as prostate, colon, skin and breast, correlated with the low serum level of 1,25-$(OH)_2D_3$, low level of dietary calcium intake or not sufficient exposure to sunlight [2–9]. Moreover, vitamin D receptor (VDR) polymorphism is correlated with a high risk of prostate cancer in men [10].

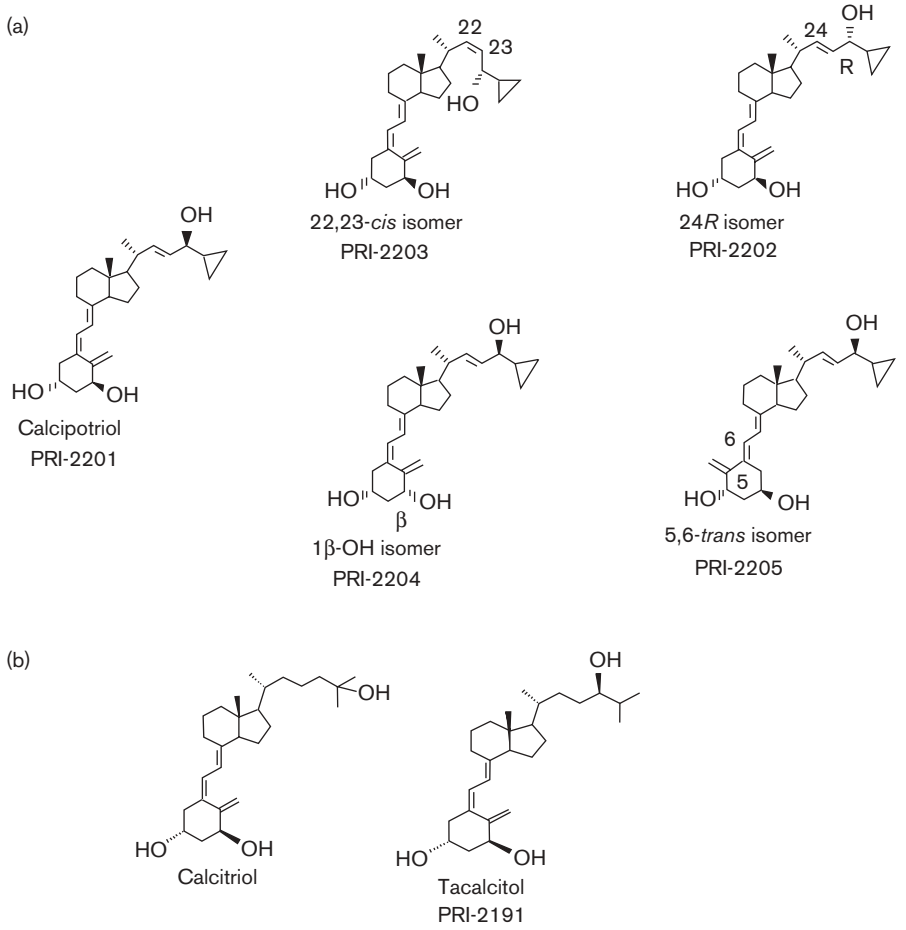
The intracellular receptor of vitamin D is a member of the steroid hormone receptor family, and it is known that the effects of calcitriol and its analogs are exerted via VDR [11,12]. After ligand activation, VDR binds specific nucleotide sequences (vitamin D response elements) in target genes with the subsequent activation or repression of their expression, most probably through multiple but not precisely defined interactions with coactivator complexes and some components of the basal transcription machinery. The presence of VDR was detected not only in the tissues related to calcium and bone metabolism, but also in a variety of cancer cells [12–14]. It was shown that growth inhibition of cancer cells caused by calcitriol requires VDR, but that this effect is modulated by nonreceptor factors that are cell line-specific [14–17].

Calcitriol and several synthetic vitamin D analogs, with reduced calcemic activity, inhibit the growth of a number of various cancer cells (of epithelial, melanoma, soft tissue sarcoma and leukemic origin) by inducing cell cycle arrest or apoptosis [18–28]. Furthermore, they inhibit the synthesis of several invasion-associated proteins [29–31], and the tumor-associated angiogenesis [24,31] of breast and colon cancer *in vivo*, and they show a chemopreventive effect on colorectal, breast and prostate cancer in laboratory animal models [32,33].

Some clinical trials with the application of analogs of calcitriol in cancer patients have been performed [32,34–37].

A serious limitation to their clinical use is hypercalcemia as the result of application of higher than physiological doses. The undesired hypercalcemia after calcitriol application explains the motivation to develop analogs, which could enable differentiation of the calcemic and

Fig. 1



Chemical structure of compounds tested. (a) Compounds tested: calcipotriol (PRI-2201), 24*R* calcipotriol (PRI-2202), 22,23-*cis* calcipotriol (PRI-2203), 1β-OH calcipotriol (PRI-2204) and 5,6-*trans* calcipotriol (PRI-2205). (b) Control compounds: calcitriol [1,25 $(OH)_2D_3$] and tacalcitol [1,24$(OH)_2D_3$, PRI-2191].

bone metabolism effects from the antiproliferative activity [38–40].

In our previous studies, a series of vitamin D_2 analogs with a highly unsaturated side chain and a series of analogs of vitamin D_3 with one or two additional hydroxyl groups in the side chain were examined for their antiproliferative effect *in vitro* against various lines of human normal and cancer cells [14,19,41–44]. Synthetic analogs of vitamin D_2 with the extended and rigid side chain, coded PRI-1906 [(24*E*)-24a-homo-(1*S*)-1,25-dihydroxyergocalciferol] and its side chain unsaturated homo-analog PRI-1907 showed antiproliferative activity higher than the parental drugs [19,41,44].

It was also shown that the vitamin D_3 metabolite, i.e. (24*R*)-1,24-dihydroxyvitamin D_3 (tacalcitol, PRI-2191) possessed higher antitumor, lower calcemic activity and lower toxicity than calcitriol [43]. Moreover, we have shown that inhibition of proliferation by this analog could be attributed to the induction of cancer cell differentiation [19,41].

Calcipotriol is a synthetic vitamin D_3 analog that binds to VDRs of epidermal cells. In-vitro studies have shown that calcipotriol exerts similar effects on cell proliferation and differentiation, but has a lower effect on calcium metabolism than calcitriol [40,45–48].

The aim of these studies was to examine a biological effect (an antiproliferative activity, the effect of phase cycle *in vitro*, toxicity and antitumor activity *in vivo*) of new vitamin D analogs with diastereomeric and geometric modifications, which are the agents obtained in a course of synthesis of calcipotriol (Fig. 1a) [49].

Materials and methods

Cells

Human SCC-25 (tongue), FaDu (pharynx), A-549 (lung), LNCaP and Du145 (prostate), HL-60 (leukemia), HL-60/MX2 (subline resistant to mitoxantron), MOLT-4, CCRF/CEM, K562, U937 (leukemias), MCF-7, T47D, MDA-MB-231 (breast), ASPC-1 (pancreas), A498 (renal) and mouse WEHI-3 and P388 (leukemias), B16 (melanoma) cancer cell lines and BALB/3T3 (mouse fibroblast) were obtained from American Type Culture Collection (Rockville, Maryland, USA). Mouse mammary adenocarcinoma 16/C was a gift from Dr I. Wodinsky (Southern Research Institute, Birmingham, Alabama, USA). HCV-29T (human urinary bladder) cell line was established in the Fibiger Institute (Copenhagen, Denmark) and obtained from Dr J. Kieler. All the cell lines are being maintained in the Institute of Immunology and Experimental Therapy (Wroclaw, Poland).

All leukemia cell lines, LNCaP and ASPC-1 cells were cultured in RPMI 1640 medium (Gibco, Paisley, UK) with 2 mmol/l L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 1.0 mmol/l sodium pyruvate, and 10% fetal bovine serum (FBS) (all from Sigma-Aldrich, Steinheim, Germany). A-549, A498, HCV29T, T47D, 16/C and MDA-MB-231 cells were cultured in RPMI 1640+Opti-MEM (1:1) (both from Gibco), MCF-7 cells in Eagle medium (IIET, Wroclaw, Poland), Du145, B16 and BALB/3T3 in Dulbecco medium (IIET) supplemented with 2 mmol/l L-glutamine and 1.0 mmol/l sodium pyruvate, and 10% FBS (all from Sigma-Aldrich). SCC-25 and FaDu cells were cultured in F-12 medium (IIET), supplemented as above.

The culture of T47D and MCF-7 cells was supplemented with 0.8 mg/l of insulin and of SCC-25 cells with 0.4 mg/l of hydrocortisone (both from Sigma-Aldrich). All culture media were supplemented with 100 units/ml penicillin, and 100 µg/ml streptomycin (both from Polfa Tarchomin, Warsaw, Poland). All cell lines were grown at 37°C with a 5% CO_2 humidified atmosphere.

Compounds

The following analogs of calcitriol were used: PRI-2191, PRI-2201, PRI-2202, PRI-2203, PRI-2204 and PRI-2205 (Fig. 1a and b). The compounds were synthesized and obtained from the Pharmaceutical Research Institute (Warsaw, Poland). Samples of the compounds were stored in amber ampoules, under argon, at −20°C. Before usage, the compounds were dissolved in absolute ethanol to the concentration of 10^{-4} mol/l and subsequently diluted in culture medium to reach the required concentrations (ranging from 1 to 1000 nmol/l). For animal experiments, compounds were dissolved in 99.8% ethanol, then diluted in 80% propylene glycol to reach the required concentrations and administered subcutaneously (s.c.) to mice in a volume 50 µl/10 g of body weight.

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated CD11b monoclonal antibodies (moAb) (clone 44) and CD14 moAb (clone UHCM-1) were purchased from Sigma (St Louis, Missouri, USA). Isotype control FITC-conjugated mouse immunoglobulins were purchased from BD Pharmingen (San Diego, California, USA). All antibodies were used at concentrations recommended by the suppliers.

An antiproliferative assay *in vitro*

At 1–2 h before addition of the tested compounds, the cells were plated in 96-well plates (Sarstedt, Germany) at a density of 5×10^3 for leukemic (with the exception of HL-60/MX2 1×10^4) and fibroblasts and 1×10^3 cells per well for the adherent cell lines. An assay was performed after 120 h exposure to varying concentrations of the tested agents.

The results were calculated as an IC_{50} (inhibitory concentration 50) – the dose of tested agent that inhibits proliferation of 50% of the cancer cell population. IC values were calculated for each experiment separately and mean values ± SD are presented in the tables. Each compound in each concentration was tested in triplicate in a single experiment, which was repeated three to seven times.

Cytotoxic test sulforhodamine B

The cells were attached to the bottom of plastic wells by fixing them with cold 50% trichloroacetic acid (Sigma-Aldrich) on the top of the culture medium in each well. The plates were incubated at 4°C for 1 h and then washed five times with tap water. The cellular material fixed with trichloroacetic acid was stained with 0.4% sulforhodamine B (Sigma-Aldrich) dissolved in 1% acetic acid (POCH, Gliwice, Poland) for 30 min. Unbound dye was removed by rinsing (4×) in 1% acetic acid. The protein-bound dye was extracted with 10 mmol/l unbuffered Tris base (POCH) for determination of the optical density ($\lambda = 540$ nm) in a computer-interfaced, 96-well microtiter plate reader Multiskan RC photometer (Labsystems, Helsinki, Finland).

MTT

Twenty microliters of MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich); stock solution: 5 mg/ml] was added to each well and incubated for 4 h. After the incubation time was complete, 80 µl of the lysing mixture was added to each well [lysing mixture: 225 ml dimethylformamide, 67.5 g sodium dodecyl sulfate (Sigma-Aldrich) and 275 ml of distilled water]. The optical densities of the samples

were read after 24 h on a Multiskan RC photometer (Labsystems) at 570 nm.

AlamarBlue assay

Twenty microliters of AlamarBlue (BioSource, Camarillo, California, USA) solution was added to each well and incubated for 6 h, the fluorescence of the samples were read on a Wallac VICTOR2 1420 multilabel counter (PerkinElmer Life Sciences, Zaventum, Belgium) at excitation 530 and emission 560 nm.

The background optical density was measured in the wells filled with culture medium, without the cells.

Cell cycle analysis

The cultured cells were seeded at a density of $1–2 \times 10^5$ cells/ml of culture medium on 24-well plates (Sarstedt) to the final volume of 2 ml. The cells were exposed to the test compound at concentrations ranging from 0.1 to 1000 nmol/l during 120 h. Ethanol as a solvent for all compounds, diluted corresponding to its highest concentration applied for the compounds, produced no toxicity. After 120 h of incubation, the cells were collected, washed in phosphate-buffered saline (PBS) and counted in a hemacytometer.

Cells (1×10^6) were washed twice in cold PBS and fixed for 24 h in 70% ethanol at -20°C. Then the cells were washed twice in PBS and incubated with RNase (50 μg/ml; Fermentas, Germany) at 37°C for 1 h. The cells were stained 30 min with propidium iodide (50 μg/ml, Sigma-Aldrich) at 4°C and the cellular DNA content was determined using the Cell Quest program (Becton Dickinson, San Jose, California, USA).

HL-60 differentiation assay

The cultured cells were seeded at a density of 2×10^5 cells/ml of culture medium on 24-well plates (Sarstedt) to the final volume of 2 ml. The cells were exposed to the study compound at concentrations in the range from 0.1 to 100 nmol/l during 120 h. Ethanol as a solvent for all the compounds tested, in dilution corresponding to its highest concentration used for the compounds, produced neither toxicity nor differentiation of HL-60 cells. After 120 h of incubation, the cells were harvested by centrifugation, washed in PBS and counted in a hemacytometer.

To determine CD11b and CD14 expression by flow cytometry, 2.0×10^5 HL-60 cells in 100 μl of PBS (without Mg^{2+}, Ca^{2+}, supplemented with 2% FBS) were mixed with an appropriate volume of moAb solution (prechilled to 4°C). The cells were incubated for 45 min in an ice bath and subsequently washed twice in 500 μl of PBS (supplemented as above). FITC-conjugated IgG1 was used as the negative control. Cell surface fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickinson). Damaged cells were labeled by adding 10 μl of propidium iodide solution (25 μl/ml) to each test tube just before data acquisition. Data analysis was performed using the WinMDI 2.8 software (The Scripps Research Institute, La Jolla, California, USA).

Animal experiments

$[C57Bl/6 \times DBA/2]F_1$ (BDF_1) male (toxicity) or C57Bl/6 female (antitumor and calcemic activity) 12–16-week-old mice, weighing 20–25 g, supplied from the Medical University Wroclaw or the Nofer Institute of Occupational Medicine, Lodz, Poland, were maintained under standard laboratory conditions. All experiments were performed according to Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education issued by the New York Academy of Sciences' Ad Hoc Committee on Animal Research and were approved by the first Local Ethics Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland.

Toxicity of new analogs

The subacute toxicity (3-week observation) after five daily s.c. administrations was determined.

Antitumor activity in the Levis lung cancer model

LLC cells derived from *in vitro* culture were inoculated s.c. into the right flank region at 3×10^5 cells per mouse (day 0).

The antitumor effect of calcitriol (2 μg/kg/day) and PRI-2201 with PRI-2202 and PRI-2205 (20 μg/kg/day) injected s.c. on days 3, 5, 7, 10, 12, 14, 17 and 19 (eight doses) was examined (experiment no. 1).

In the following experiment (no. 2), the antitumor activity of PRI-2201 with PRI-2205 applied in various doses (1, 10, 50 and 100 μg/kg/day) injected s.c. on days 3, 5, 7, 10, 12, 14 and 17 (seven doses) was evaluated.

Calcemic activity

The serum calcium level was determined in all mice bearing LLC tumors from the first experiment and from other experiments involving mice treated with 50 or 100 μg/kg/day of the tested compounds and compared with the level of control mice.

The calcium level was measured in each individual serum sample with the photometric Arsezano 3 method (Olympus AU400; Olympus America, Melville, New York, USA).

Evaluation of the antitumor effect *in vivo*

The tumor volume was calculated using the formula $(a^2 \times b)/2$, where a is the shorter diameter in millimeters

Table 1 Antiproliferative activity *in vitro* (ID_{50}) of calcitriol and its analogs against sensitive cancer cell lines and normal fibroblasts (BALB/3T3)

Compound	Cell line ID_{50}(nmol/l) (mean ± SD)						
	SCC-25	MCF-7	T47D	HL-60/MX2	HL-60	WEHI-3	BALB/3T3
Calcitriol	134.7 ± 18.8	43.6 ± 14.3	67.7 ± 20.8	1000.0 ± 0.0	46.7 ± 13.1	5.4 ± 2.3	70.6 ± 52.8
PRI-2191	42.1 ± 25.5*	19.6 ± 14.7*	23.8 ± 15.5*	11.8 ± 8.8*	13.1 ± 1.6	5.2 ± 0.4	5.4 ± 5.9*
PRI-2201	57.5 ± 21.5	14.9 ± 12.0*	38.7 ± 27.5	7.2 ± 2.4*	6.0 ± 3.4	9.5 ± 4.9	24.8 ± 25.7
PRI-2202	218.1 ± 41.8	137.7 ± 98.3*	172.3 ± 14.5*	139.2 ± 53.0*	98.9 ± 4.55*	31.2 ± 2.1*	268.6 ± 80.2*
PRI-2203	270.3 ± 29.1*	413.1 ± 158.4*	651.9 ± 263.4*	590.0 ± 235.6*	164.5 ± 13.5*	76.0 ± 29.2*	405.4 ± 37.0*
PRI-2204	506.6 ± 260.4*	525.4 ± 148.1*	> 1000	300.1 ± 40.6*	159.8 ± 28.0*	370.5 ± 43.6*	960.1 ± 263.4*
PRI-2205	534.1 ± 216.3*	46.9 ± 8.6	410.4 ± 127.4*	4.6 ± 2.6*	24.0 ± 12.9*	38.8 ± 4.2*	63.5 ± 19.0

*$P < 0.05$ Mann–Whitney *U*-test (compound tested vs. calcitriol).

Table 2 Expression of CD11b and CD14 cell-surface markers on HL-60 cells exposed to $1,25(OH)_2D_3$ or its analogs PRI-2191, PRI-2201, PRI-2202 and PRI-2205 (10 nmol/l) for 120 h

Compound	Dose (nmol/l)	CD14		CD11b	
		(%) ± SD	GeoMC ± SD	(%) ± SD	GeoMC ± SD
Ethanol	0.01%	8.29 ± 6.11	4.65 ± 2.18	3.84 ± 3.37	3.17 ± 0.57
	0.1%	4.49 ± 3.34	4.2 ± 0.9	1.32 ± 1.18	3.5 ± 1.1
Calcitriol	10	74.84 ± 10.02*	14.83 ± 9.18	40.33 ± 12.57*	5.54 ± 1.25*
	100	74.97 ± 15.29*	19.5 ± 1.7*	62.77 ± 21.89*	12.0 ± 4.7*
PRI-2191	10	80.52 ± 8.24*	18.35 ± 12.95*	46.01 ± 6.25*	6.14 ± 1.72*
	100	77.06 ± 14.11*	21.3 ± 0.4*	65.56 ± 19.09*	12.4 ± 5.0*
PRI-2201	10	68.89 ± 12.71*	14.96 ± 12.44	40.84 ± 15.30*	5.25 ± 1.3
	100	76.56 ± 14.66*	20.9 ± 0.8*	60.13 ± 23.51*	12.0 ± 6.7*
PRI-2202	10	7.01 ± 5.90	4.57 ± 2.1	6.79 ± 7.15	2.97 ± 0.22
	100	67.54 ± 31.50*	8.8 ± 1.3	37.56 ± 17.96*	7.3 ± 3.4
PRI-2205	10	16.45 ± 7.42	5.56 ± 2.93	15.09 ± 9.68*	3.66 ± 0.79
	100	56.53 ± 25.85*	8.4 ± 1.1*	36.75 ± 15.11*	7.0 ± 2.9*

Results of four to six experiments are presented as percentage of the positive cell (%) and mean channel (MC) of fluorescence of the positive cell population (± SD). Percentage of positive cells was calculated by subtraction of a common area under graphs (graph representing negative control and graph representing an expression of antigen) from an area under the graph representing the expression of antigen.
*Differ significantly from control (Mann–Whitney *U*-test).

and *b* is the longer diameter in millimeters. Inhibition of tumor growth was calculated from the following formula: TGI (%) (tumor growth inhibition) = $(W_T/W_C) \times 100 - 100\%$, where W_T and W_C are the median tumor weights of treated mice and untreated control animals, respectively. The animals were killed 20 days (experiment no. 1) or 18 days (experiment no. 2) after inoculation of the LLC cells, and blood sera were collected. Internal organs were examined macro- and microscopically.

Statistical evaluation

The Mann–Whitney *U*-test and one-way analysis of variance followed by the Tukey HSD test were applied. A *P*-value less than 0.05 was considered significant.

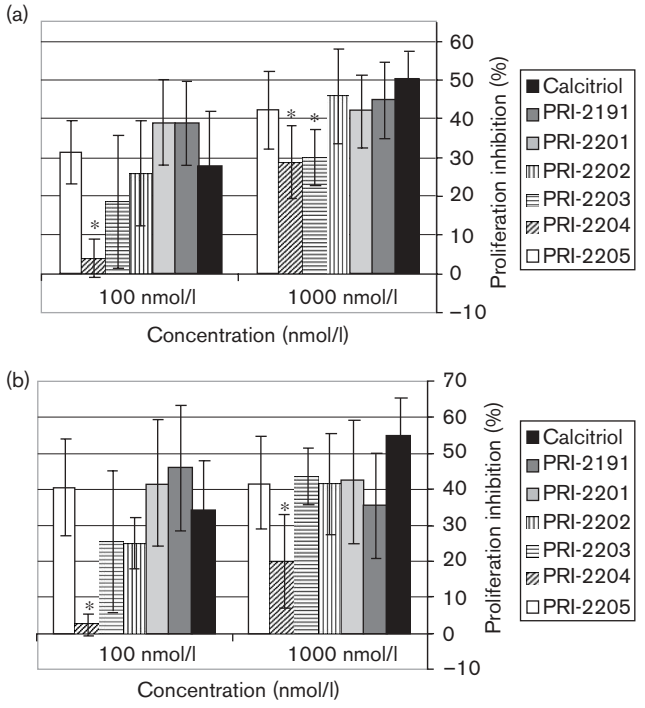
Results

Antiproliferative activity of vitamin D_3 analogs

All compounds were examined for their antiproliferative activity *in vitro* against the cells of human and mouse cancer cell lines, and against mouse fibroblasts. The analogs tested revealed the highest antiproliferative activity against the following cancer cell lines: T47D, MCF-7, SCC-25, HL-60, HL-60/MX, WEHI-3 and against mouse fibroblast BALB/3T3 with maximal inhibition of proliferation above 50% (Tables 1 and 2). This effect was dose-dependent. For the cell lines mentioned above, the IC_{50} values were calculated and compared with those of referential calcitriol and tacalcitol (PRI-2191) (Table 1). It appeared that PRI-2201 (calcipotriol) revealed the strongest (comparable to that achieved with PRI-2191) antiproliferative effect among all new analogs tested. This effect was about 2–140 times stronger than that of calcitriol (Table 1). Moreover, when the effect against individual cell lines is considered, the analog PRI-2205 revealed a marked effect against HL-60, HL-60/MX2 and BALB/3T3. The activity of this analog was comparable or even higher than calcitriol. PRI-2202 was a less-active analog than calcitriol, but still revealed activity comparable to PRI-2205, especially against SCC-25, T47D or WEHI-3 cell lines.

A similar profile of activity was observed for the FaDu and 16/C cell lines, which were less sensitive to the antiproliferative effect of calcitriol analogs (Fig. 2). Data for other less sensitive (inhibition of proliferation caused by dose of 1000 nmol/l ranged from 20 to 50%) cell lines (LNCaP, CCRF/CEM, A549, K562, MDA-MB-231, ASPC-1 and Du145) or below 20% (U937, HCV29T, A498, P388, L1210 and B16) are not shown.

Fig. 2



The antiproliferative effect *in vitro* of vitamin D analogs against cells of human pharynges squamous carcinoma cell line FaDu (a) and mouse mammary cancer cell line 16/C (b). *Indicates statistically significant ($P \leq 0.05$, Mann–Whitney *U*-test) (all compounds vs. calcitriol).

Table 3 The effect of exposure of HL-60 cells to either calcitriol or its analogs on apoptosis and cell cycle distribution

Compound	Dose (nmol/l)	Cell cycle distribution (mean% ± SD)			
		M1	M2	M3	M4
Ethanol	0.01%	55.5 ± 5.5	16.6 ± 3.1	16.2 ± 4.0	9.6 ± 4.2
	0.1%	55.5 ± 4.9	17.4 ± 1.7	17.5 ± 4.7	9.8 ± 6.2
Calcitriol	10	64.2 ± 6.8[a]	12.3 ± 3.1[a]	12.4 ± 4.2	11.6 ± 8.7
	100	78.2 ± 2.9[a]	7.9 ± 1.4[a]	11.0 ± 1.5	2.9 ± 2.6
PRI-2191	10	68.2 ± 6.5[a]	10.5 ± 2.0[a]	12.2 ± 3.4[a]	9.4 ± 8.6
	100	80.4 ± 4.0[a]	7.0 ± 2.0[a]	9.9 ± 2.8[a]	2.8 ± 2.0
PRI-2201	10	65.1 ± 6.5[a]	11.2 ± 2.3[a]	13.0 ± 4.3	11.0 ± 8.5
	100	79.3 ± 3.8[a]	7.8 ± 1.7[a]	11.1 ± 2.7	2.0 ± 0.8[a]
PRI-2202	10	51.1 ± 8.2	15.9 ± 4.7	14.0 ± 5.1	19.4 ± 10.2[a]
	100	67.5 ± 2.4[a]	13.2 ± 1.7[a]	14.0 ± 2.6	5.6 ± 1.6
PRI-2205	10	54.8 ± 5.1	15.5 ± 4.4	14.1 ± 4.3	15.9 ± 7.9[a]
	100	72.8 ± 3.0[a]	10.7 ± 1.6[a]	13.9 ± 1.9	2.8 ± 2.0

The results are presented as a mean percentage of the cell population qualified to one of the four groups: M1 cells in phase G0/G1, M2 cells in phase S, M3 cells in phase G2/M and M4 apoptotic cells.
[a]Indicates statistically significant ($P \leq 0.05$) (tested compounds vs. ethanol).

Expression of CD11b and CD14 cell-surface markers on HL-60 cells exposed to calcitriol or PRI-2191, PRI-2201, PRI-2202 and PRI-2205 for 120 h

HL-60 cells were exposed during 120 h to either calcitriol or PRI-2191, PRI-2201, PRI-2202 or PRI-2205 at various concentrations. When the incubation was completed, the presence of cell-surface differentiation markers was analyzed. Control untreated HL-60 cells showed a very low expression of CD11b and CD14 cell-surface markers. After exposure to either calcitriol, PRI-2191 or PRI-2201, the expression of those cell-surface markers characteristic for monocytes, markedly increased in a concentration-dependent mode. The determined antigen CD14 and CD11b expression after exposure to calcitriol and its new analogs confirmed their effect on cell differentiation. In the case of analogs PRI-2202 and PRI-2205, the increase of monocyte-specific surface markers was very weak and was only statistically confirmed at the concentration of 100 nmol/l (Table 2).

Cell cycle analysis of human leukemia cell lines HL-60 and CCRF/CEM

A statistically significant increase in the percentage of HL-60 cells in the G_0/G_1 cell cycle stage induced by calcitriol, PRI-2191 and PRI-2201, and cells in apoptosis induced by PRI-2202 and PRI-2205 was observed for samples of cells exposed to 10 nmol/l of these analogs. In addition, an increase of cells in the S phase was observed after exposure to 10 nmol/l of calcitriol, PRI-2191 and PRI-2201. At 100 nmol/l, the increase in the percentage of cells in the G_0/G_1 phase and the decrease of the S phase was observed also for cells exposed to PRI-2202 and PRI-2205 analogs (Table 3).

A statistically significant increase of the percentage of CCRF/CEM cells in the G_2/M stage induced by 1000 nmol/l PRI-2205 was observed (14.4 ± 2.6 for control and $20.6 \pm 5.0\%$ for PRI-2205). Similar, but not statistically significant changes in the cycle phase after exposure of cells to analog PRI-2202 (percentage of cells in G_2/M stage: $20.2 \pm 5.1\%$) were observed.

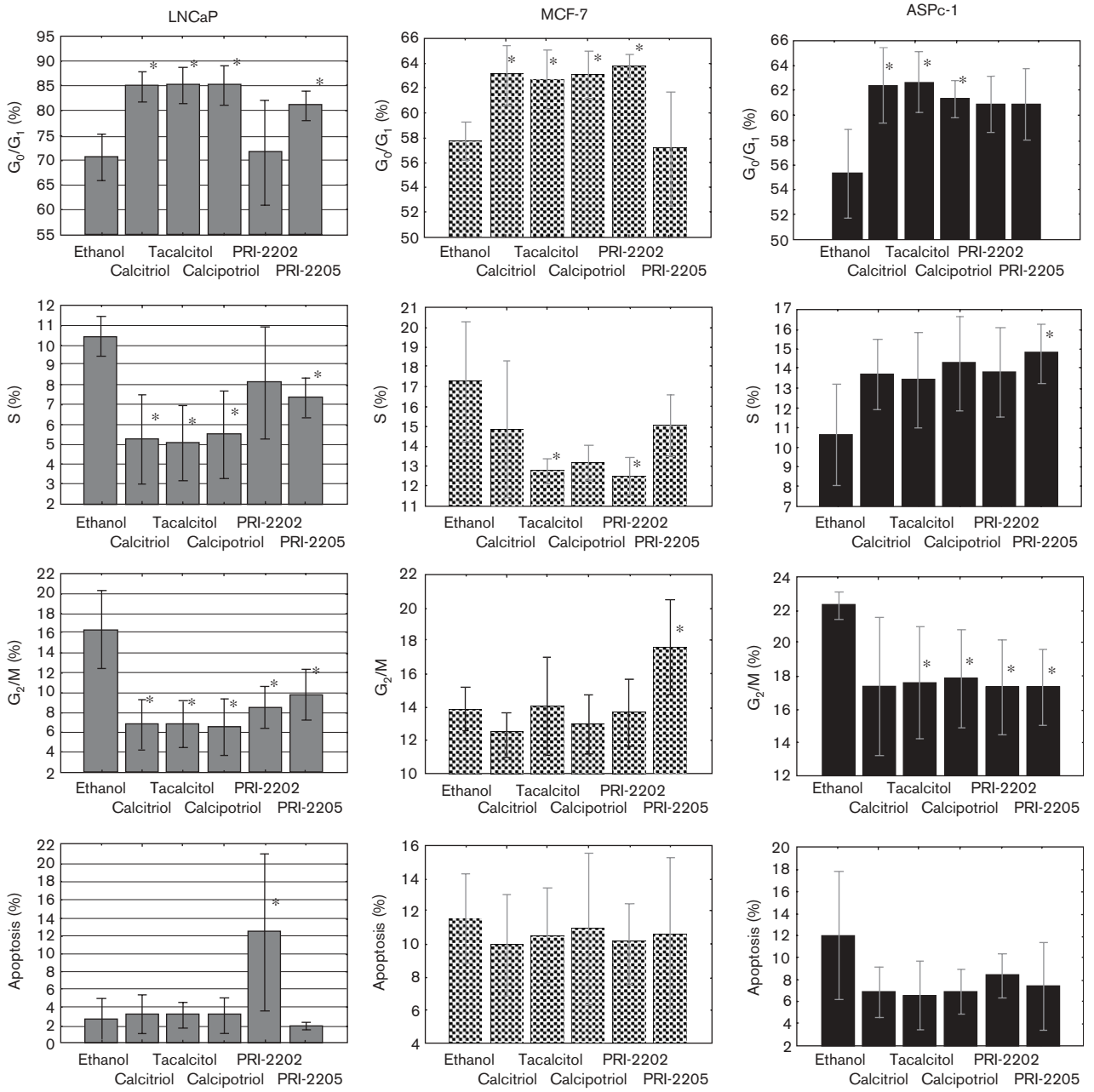
Human prostate cancer cell lines LNCaP and Du145

A statistically significant increase of percentage of LNCaP cells in the G_0/G_1 stage induced by calcitriol, PRI-2191, PRI-2201 and PRI-2205 cells was observed with a consequent decrease in the percentage of cells in S and G_2M phase. On the contrary, after incubation with analog PRI-2202 the percent increase of LNCaP cells in apoptosis and decrease of S phase was observed (Fig. 3). No effect on cell cycle in the case of the Du145 cell line at 100 or 1000 nmol/l concentration was observed (data not shown).

Human breast cancer cell line MCF-7

A statistically significant increase in the percentage of MCF-7 cells in the G_0/G_1 stage induced by calcitriol, PRI-2191, PRI-2201 and PRI-2202 and cells in G_2M phase induced by PRI-2205 was observed after exposure to 100 nmol/l of this analog. In addition, an increase in the S phase was observed after incubation with 100 nmol/l of calcitriol, PRI-2191, PRI-2201 and PRI-2202 (Fig. 3).

Fig. 3



The effect of exposure of LNCaP, MCF-7 and ASPc-1 cells to either calcitriol or its analogs. The compounds were dissolved in absolute ethanol to the concentration of 10^{-4} mol/l and subsequently diluted in culture medium to reach the required concentrations. The cultured cells were seeded at the density of $1–2 \times 10^5$ cells/ml of culture medium on 24-well plates to the final volume of 2 ml. The cells were exposed to the tested compound at concentrations: LNCaP, ASPc-1 : 1000 nmol/l, MCF-7 : 100 nmol/l during 120 h. Ethanol as a solvent for all compounds, diluted corresponding to their concentration was used. The results are presented as mean percentage of the cell population qualified to one of the cell cycle phases: M1 – G_0/G_1, M2 – S, M3 – G_2/M and M4 – apoptotic cells. *Statistically significant ($P \leq 0.05$) (tested compounds vs. ethanol).

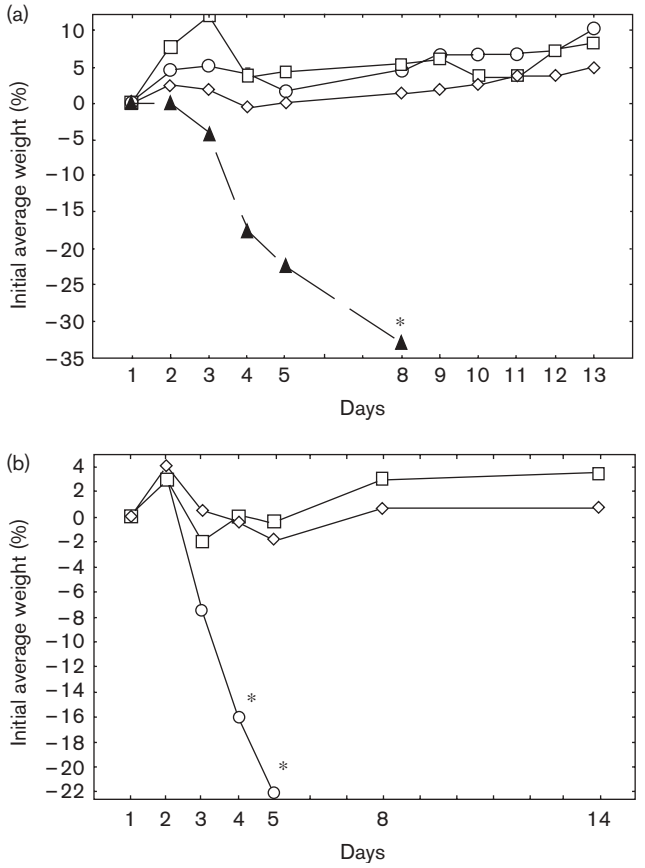
Human pancreatic cancer cell line ASPC-1

An increase in the percentage of ASPC-1 cells in the G_0/G_1 stage induced by all compounds tested was observed at a concentration of 1000 nmol/l. In addition, an increase in the S phase and a decrease in the G_2/M phase was observed after incubation with this concentration of analogs (Fig. 3). The concentration of 100 nmol/l did not affect cell division distribution (data not shown).

Toxicity of new analogs

Following on from our previous observations [43], initially we used the maximal dose of tested agents 100 μg/kg/day

Fig. 4



Kinetics of body weight in mice treated subcutaneously (s.c.) with (a) 10 μg/kg/day of calcitriol, or 100 μg/kg/day of PRI-2201, PRI-2202 or PRI-2205. *Statistically significant difference in body weight ($P<0.05$, ANOVA, Tukey HSD test): calcitriol vs. PRI-2201, PRI-2202 or PRI-2205. (b) 1000 μg/kg/day of PRI-2201, PRI-2202 or PRI-2205. *Statistically significant difference in body weight ($P<0.05$, ANOVA, Tukey HSD test): PRI-2201 vs. PRI-2202 or PRI-2205. ▲, calcitriol; ○, PRI-2201; □, PRI-2202; ◇, PRI-2205. Compounds were dissolved in 99.8% ethanol, then diluted in 80% propylene glycol to reach the required concentrations, and administered s.c. to mice (male BDF1) in a volume 5.0 μl/1.0 g of body weight during 5 consecutive days. Survival analysis (Cox–Mantel test) shows statistically significant difference between life span of control mice treated with (a) calcitriol (mean ± SD: 7.4 ± 1.1, median: 8.0 days) or (b) PRI-2201 (mean ± SD: 9.0 ± 1.0, median: 9.0 days) vs. all other respective treated groups in which all mice survived ($P<0.05$).

(and two lower doses) administered during 5 consecutive days. Calcitriol was used at the doses of 1, 5 and 10 μg/kg/day.

Even at the highest dose, no toxic death and no body weight decrease was observed in the case of the new analogs tested (Fig. 4a). Calcitriol used as a referential control agent caused a decrease (25%) of initial body weight in mice treated with 1 and 5 μg/kg/day. All mice receiving 10 μg/kg/day of calcitriol have succumbed (Fig. 4a).

In following experiment, the doses 1000 μg/kg/day of analogs PRI-2201 and PRI-2205, and 500 μg/kg of analog PRI-2202 were used. All mice treated with PRI-2201 in this dose died after day 8 of the experiment (Fig. 4b). No significant decrease in body weight of mice was observed after treatment with two other analogs (Fig. 4b).

Antitumor and calcemic activity

The antitumor activity of these analogs in the LLC mice tumor model was tested. As shown in Table 4, the antitumor effect of PRI-2202 and PRI-2205 is higher than that for calcitriol or calcipotriol. Statistically significant inhibition of tumor growth in mice receiving PRI-2205 was observed both on the 14th and 20th day of the experiment. A similar effect is shown for the analog PRI-2202.

When the calcemic activity of these compounds was analyzed, a statistically significant increase of the calcium level in serum of mice treated with calcitriol was observed. Calcipotriol, PRI-2202 and PRI-2205 did not significantly influence the serum calcium level (Table 4).

The calcipotriol activity was compared with the activity of PRI-2205 in a similar experimental protocol, but with the application of various doses (1, 10, 50 and 100 μg/kg/day). Only a dose of 10 μg/kg/day of PRI-2205 appeared to be effective in inhibiting LLC tumor growth (Fig. 5). On day 17 of the experiment, 59% of tumor growth inhibition (TGI) by PRI-2205 was noted ($P < 0.05$) (Fig. 5). Only 21% TGI was observed when PRI-2201 was applied (data not shown). With a dose of 1 μg/kg/day of PRI-2205 the TGI was 37% and treated with PRI-2201 the TGI was 23% (not statistically significant). For the doses 50 and 100 μg/kg/day, the TGI value ranged from 0 to 23% for both analogs (data not shown).

Analyzing the calcemic activity of tested analogs only in the highest dose of 100 μg/kg/day, a statistically significant increase of serum calcium level caused by PRI-2201 and PRI-2205 was observed. The serum calcium level increased more in the case of PRI-2201 than PRI-2205 ($P < 0.05$) (Table 5).

Discussion

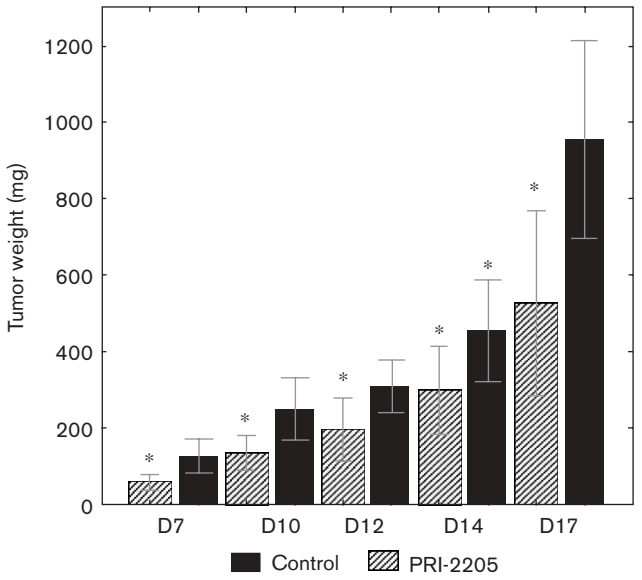
Calcipotriol (1,24-dihydroxy-22-ene-24-cyclopropyl-vitamin D_3) is an example of an active, but low-toxicity synthetic analog of calcitriol used in topical treatment of psoriasis [47]. Evidence exists that calcipotriol binds to VDRs in epidermal cells, stimulates differentiation of epidermal and leukemia cells, and has antiproliferative effects against epidermal, squamous cancer, breast cancer and leukemic cells *in vitro* [40,46–48,50–55]. Calcipotriol has been shown to inhibit the growth of breast cancer in rats. It revealed a 100–200 times lower effect on calcium metabolism than calcitriol [40,47,48,52].

Table 4 **Serum calcium level and tumor weight**

Parameter tested	Control	Calcitriol	PRI-2201	PRI-2202	PRI-2205
Calcium day 20					
Mean + SD (mEq/l)	5.10 ± 0.07[a]	8.06 ± 0.56[b,c]	5.62 ± 0.38[a,c]	5.09 ± 0.20[a]	4.96 ± 0.14[a]
Tumor day 20					
Median (g)	4.40	3.55	3.70	2.60[b]	2.55[b]
TGI (%)	–	19	16	41	42
N (day 20)	5	6	6	4	6
Tumor day 14					
Median (g)	1.37	0.68	0.95	0.73	0.67*
TGI (%)	–	46	25	43	47
N (day 14)	8	6	6	6	6

N, number of mice.
Mice bearing LLC tumors were inoculated subcutaneously with calcitriol at the dose of 2 μg/kg/day or PRI-2201, PRI-2202 and PRI-2205 at the dose of 20 μg/kg/day on days 3, 5, 7, 10, 12, 14, 17 and 19 (× 8) after tumor cells injection.
[a]$P < 0.05$ as compared to calcitriol.
[b]$P < 0.05$ as compared to control.
[c]$P < 0.05$ as compared to PRI-2205.
Statistical analysis: calcium level: one-way ANOVA (Tukey HSD Test); tumor weight: Mann–Whitney *U*-test.

Fig. 5



Kinetics of LLC tumor growth in control and mice treated with PRI-2205 at the dose of 10 μg/kg/day. *$P < 0.05$ Mann–Whitney *U*-test. LLC cells derived from *in vitro* stock culture were inoculated s.c. into the right flank region with 3×10^5 cells per mouse on day 0. PRI-2205 was applied at the dose of 10 μg/kg/day, injected s.c. on days 3, 5, 7, 10, 12, 14 and 17 (7 ×).

In these studies, we show the antiproliferative activity of four new analogs of calcitriol, produced during synthesis of calcipotriol (PRI-2201). Of them, PRI-2204 (1β-OH calcipotriol) and PRI-2203 (22,23-*cis* calcipotriol) revealed a lower effect on the inhibition of proliferation of cells of various cancer cell lines in comparison with the analog PRI-2202 (24*R* calcipotriol) and PRI-2205 (5,6-*trans* calcipotriol). The activity of some of these analogs is similar, but in some models *in vitro* as much as seven times lower than calcitriol. Tacalcitol and calcipotriol appeared to be the most biologically active agents (Table 1).

The mechanism of the antiproliferative effect of calcitriol and its analogs *in vitro* is related to their effect on cell differentiation [39,47,51,53,55–58]. Studies using cancer cell lines showed that calcitriol forced cancer cells to accumulate in the G_0/G_1 [18–20,41,58,59] phase of the cell cycle or in the G_2M [59,60]. Deregulation of intracellular signal transduction and/or induction of apoptosis may be also considered [20,28]. Three compounds, i.e. calcipotriol, PRI-2202 and PRI-2205, used in the same experiment with referential agents calcitriol and PRI-2191 were analyzed also in respect to their effect on differentiation of HL-60 cells and their influence on distribution of cell cycle phases of cells from HL-60, CCRF/CEM, LNCaP, Du145, ASPC-1 and MCF-7 cell lines. In general, induction of differentiation by calcitriol, PRI-2191 and PRI-2201 was observed. The cells cumulate in the G_0/G_1 stage. The analogs PRI-2202 and PRI-2205 appeared to be less potent in induction of cancer cells differentiation. They caused apoptosis of HL-60 cells at a dose of 10 nmol/l, but in the higher dose (100 nmol/l) caused cell differentiation (Tables 2 and 3). Prostate cancer cells LNCaP cumulated in the G_0/G_1 stage after incubation with PRI-2205, but PRI-2202 caused apoptosis of these cells (Fig. 3). In the case of MCF-7 breast cancer and CCRF/CEM leukemia cell lines, however, analog PRI-2205, in contrast to all other analogs, increased accumulation of cells in G_2/M stage (Fig. 3). Moreover, in the case of the pancreatic cancer cell line ASPC-1, contrary to all other cell lines used, an increase of cells in the S stage after incubation with all compounds was observed (Fig. 3).

Interestingly, the toxicity studies showed that PRI-2202 and PRI-2205 appeared to be the less toxic analogs. Even in total doses of 2.5–5.0 mg/kg (distributed during 5 successive days), no changes in body weight were observed. Calcitriol and tacalcitol in the same protocol showed toxicity at 100 times lower doses. Total LD_{50} for calcitriol was 37 and for tacalcitol was 105 μg/kg [43]. Also, calcipotriol caused death of all mice (mean

Table 5 Serum calcium level in mice injected s.c. with PRI-2201 or PRI-2205

	Control	PRI-2201 (μg/kg/day)		PRI-2205 (μg/kg/day)	
		50	100	50	100
Calcium (mEq/l)	4.77 ± 0.10	5.11 ± 0.25	5.73 ± 0.37[a,b]	5.01 ± 0.18	5.15 ± 0.26[a]
N	11	5	6	5	5

[a]$P < 0.05$ as compared to control.
[b]$P < 0.05$ as compared to PRI-2205 (100 μg/kg/day).
One-way ANOVA (Tukey HSD Test).

life-span ± SD: 7.4 ± 1.1 days) when the total dose of 5.0 mg/kg was administered (Fig. 4b).

The antitumor activity of these analogs tested in the LLC mice tumor model shows that the analog PRI-2205 appears to be more effective than calcitriol or calcipotriol and PRI-2202 (Table 4). Our unpublished results from an experiment in which mice bearing LLC cancer transplanted s.c. were administered orally with calcitriol or tacalcitol (PRI-2191), showed statistically significant TGI (applied according to following schedule: 5 μg/kg/day in days 3, 6, 8, 10, 13 and 15, ×6). TGI was 51% (calcitriol) and 42% (tacalcitol). Moreover, PRI-2205 revealed no calcemic activity at the doses that inhibit tumor growth or applied in higher doses.

These data demonstrate that the analogs PRI-2202 and PRI-2205 are nontoxic and potent inhibitors of cancer cell proliferation *in vitro* and cancer growth *in vivo*. In particular, their effect in a combined treatment protocol with cytostatics should be considered for further study.

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